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Open-state substructure of single chloride channels from *Torpedo* electroplax

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Chloride channels from *Torpedo californica* electroplax were inserted into planar phospholipid membranes, and single-channel currents were studied at high time-resolution. The open channel fluctuates rapidly between three substates, with conductances of 18.5, 9.4 and 0 pS in 150 mM Cl^-. Under various ionic conditions the three substates are always equally spaced in conductance; at various voltages leading to different probabilities of observing the three substates, the substate frequencies are always binomially distributed. The conclusion emerges that the conducting unit of this Cl^- channel is composed of two identical Cl^- diffusion pathways, each with a voltage-dependent gate.

Introduction

The electric marine ray *Torpedo californica* is considered by membrane biochemists to be a swimming purified acetylcholine receptor, and it is indeed true that the electroplax organ of this animal is an extremely rich source of the receptor protein. But recent work on the fusion of electroplax membrane vesicles with planar lipid bilayers (White & Miller 1979, 1981*a*; Miller & White 1980) and on Cl^- fluxes in these vesicles (Taguchi & Kasai 1980; White & Miller 1981*b*) has shown that the electric organ also contains a voltage-dependent Cl^- specific channel. Purification studies suggest that these Cl^- channels reside in the non-innervated-face plasma membrane of the electroplax cell, not in the acetylcholine receptor-rich innervated-face membrane (White 1981).

The electrical properties of the Cl^- channel have been characterized in planar phospholipid bilayers into which electroplax membrane vesicles have been fused. The channel is unusually Cl^--selective: of all other ions tested, only Br^- shows any measurable permeability (Miller & White 1980); the channel is reversibly blocked in a voltage-dependent manner by SCN^- (White & Miller 1981*a*), and is inhibited asymmetrically by stilbene disulphonates (White & Miller 1979). The channel conductance saturates with increasing Cl^- concentration, as expected for a single-ion conduction mechanism (Läuger 1973) in which at most a single Cl^- ion may occupy the channel; the maximum single-channel conductance is about 30 pS, and the half-saturation Cl^- concentration is 100 mM. Previous work showed that a simple two-state 'open–closed' mechanism appeared to provide an adequate description of the voltage-dependent gating process. The opening and closing kinetics were found to be quite slow, in the 1–10 s timescale. The general conclusion emerging from this previous study was that conventional models of ion channel gating and conduction known to apply to a variety of cation-selective channels also apply to the *Torpedo* Cl^- channel, which is the only anion-specific channel so far accessible to detailed investigation.

This report describes for this Cl^- channel a type of single-channel behaviour utterly

unprecedented in any other known channel. These new results, which required the improvement of the electrical recording system, demonstrate that the channel, once open, fluctuates rapidly between three distinct 'substates'. The detailed behaviour of these substate fluctuations argues strongly that the conducting unit of the channel is a structure consisting of *two* identical Cl^- diffusion pathways, each containing a voltage-dependent gate.

Materials and methods

Biochemical

Vesicles from *Torpedo* electroplax enriched in non-innervated-face membranes were prepared as described by White & Miller (1981*b*) immediately after dissection of the electric organ. Lipids used to form bilayers were bovine phosphatidylethanolamine (PE) and phosphatidyl-

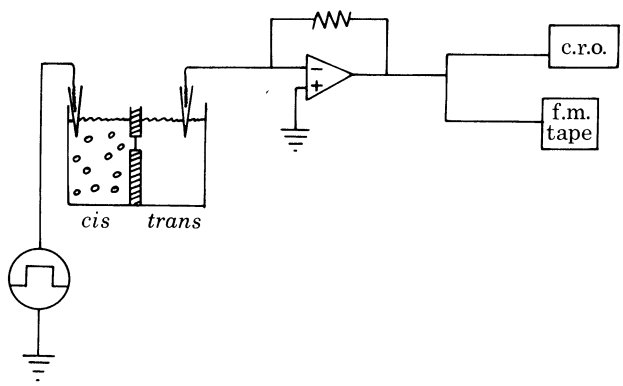


FIGURE 1. Schematic of experimental system. The planar bilayer system consists of two aqueous chambers, the *cis* side, to which *Torpedo* membrane vesicles are added, and the *trans* side, which is by convention defined as zero voltage. Command voltage is supplied to the *cis* chamber by a pulse generator or a variable battery.

serine (PS), purified as described by Labarca *et al.* (1980); in some experiments, the same lipids were purchased from Avanti Polar Lipids, Birmingham, Alabama, and gave results identical to those obtained with the home-purified samples. (This was not so for commercial lipids from other sources.) Except where indicated otherwise, the aqueous solution used in the bilayer system consisted of 150 mM KCl, 20 mM HEPES and 0.1 mM EDTA, adjusted to pH 7.4 with KOH.

Planar bilayers and vesicle fusion

The planar bilayer system has been described in detail (Miller 1978, 1982) and is shown schematically in figure 1. To obtain the higher sensitivity and time-resolution required for these experiments, a low voltage-noise operational amplifier (LF157A, National Semiconductor) was used, combined with compensation to reduce the effective capacitance of the 10 GΩ feedback resistor to 60 fF. Furthermore, low-capacitance planar bilayers (80–150 pF) were formed by casting membranes from 50 mM lipid solutions in *n*-decane onto a 100 μm diameter hole in a polystyrene septum. In all experiments, the lipid mixture was 70% PE/30% PS.

Fusion of vesicles with the planar bilayer was accomplished by addition of 1–5 μg ml^{-1} of *Torpedo* membranes (loaded with 400 mM sucrose) to the '*cis*' side of the bilayer, in the presence

of 0.8 mM $CaCl_2$ also added to that side. Bilayer conductance was monitored continuously at +35 mV applied voltage (referred to the '*trans*' side of the membrane, opposite to the addition of vesicles). Immediately after the first fusion event (observed as an abrupt increase in bilayer conductance), 1.2 mM EDTA was added to inhibit further channel incorporation, the stirrer was shut off, and records were collected on f.m. tape. To ensure that only a small number of channels were inserted per fusion event, the vesicles were sonicated for 2 min in a bath sonicator before use.

Results

The small number of single-channel data reported on this system previously (Miller & White 1980; White & Miller 1981*a*) were obtained by using an amplifier of low time-resolution (0.3 s); clean channel fluctuations could be observed at voltages more positive than +20 mV, but at

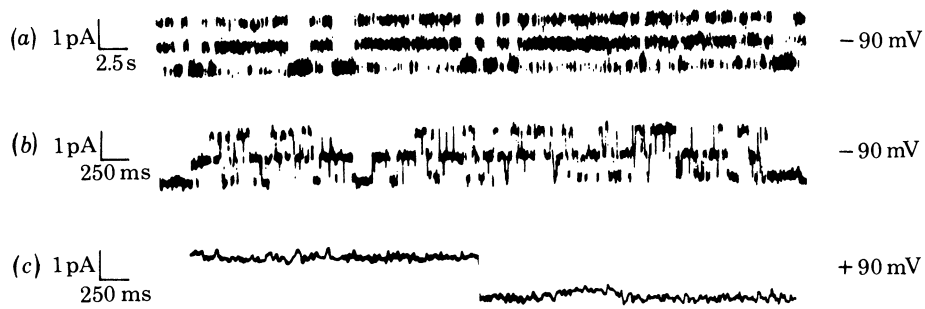


FIGURE 2. Single Cl^- channels at high time-resolution. A single Cl^- channel was incorporated into a PE/PS bilayer as described in the text, and records were taken, with low-pass filtering at 600 Hz cutoff frequency. (*a*) Long records of opening and closing of the channel, at −90 mV. (*b*) Expanded timescale record of a single opening event at −90 mV. (*c*) Channel closing event at +90 mV; for this trace, the channel was opened with a 1 s pulse to −90 mV, and then the test voltage of +90 mV was applied to favour channel closing. An upward deflexion always represents increased conductance.

negative voltages it was found that the channels were unaccountably 'noisy.' Now, by using an amplifier of 200-fold higher time-resolution, it is possible to observe the channel fluctuations over the entire voltage range (figure 2). At negative voltage (−90 mV in this example), the channel is seen to open and close slowly when examined on a timescale of seconds (trace *a*); at this highly negative potential, the channel is only rarely in its closed state, as expected from previous work (White & Miller 1979; Miller & White 1980). However, we now see that the open channel does not merely display a single open state. Instead (trace *b*), once open the channel fluctuates rapidly between *three* well defined 'substates' with approximate conductances of 20, 10 and 0 pS at this Cl^- concentration. Throughout this paper I shall refer to these substates as the U, M and D states, respectively. It is also important to realize that the short-lived zero-conductance D state is clearly different from the long-lived, zero-conductance intervals between channel openings; I shall refer to these intervals as the 'closed' state of the channel. In trace *c*, the open channel is examined at a highly positive voltage, +90 mV. Here, in marked contrast to its behaviour at −90 mV, only a single open state is observed, of approximately 20 pS conductance, i.e. the conductance of the U state.

The substructure of the channel's voltage dependence is examined more closely in figure 3,

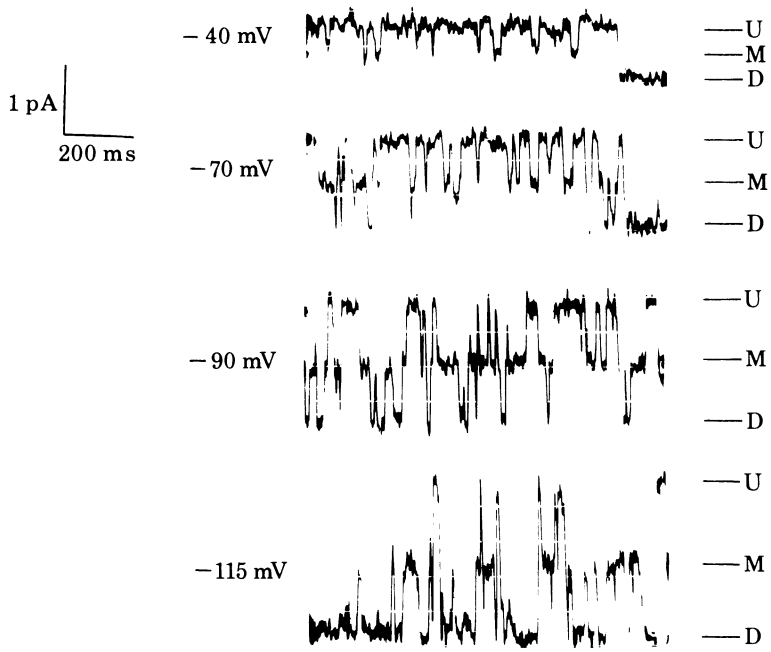


FIGURE 3. Voltage dependence of open channel substructure. A single Cl^- channel was inserted into a bilayer as in figure 2, and records were collected at the holding potentials indicated. Traces displayed are all taken from within an open-state duration, i.e. channel closing events are not shown. Lines mark the current levels corresponding to the U, M and D states of the open channel. Note the closing of the channel at the ends of the top two traces.

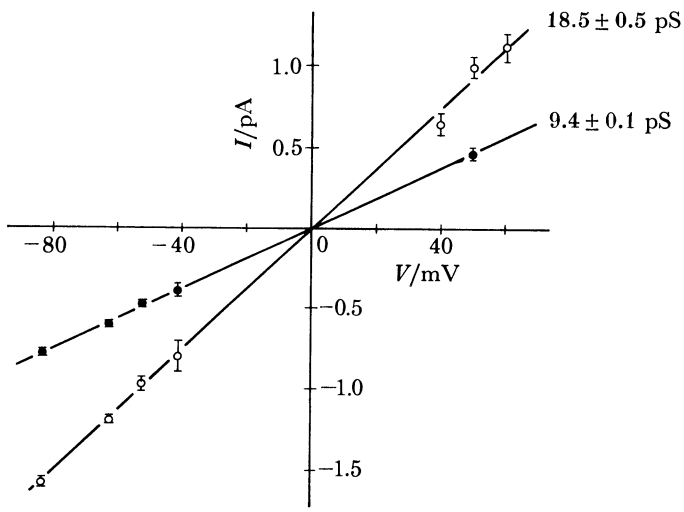


FIGURE 4. Current–voltage relation of open-channel substates. Single-channel substate records were collected at various voltages, and the currents corresponding to the U state (open points) and M state (filled points) were measured by hand. Each point represents the mean ± s.e.m. of 5–20 determinations. Current was always measured with respect to the closed state of the channel. The D-state current was indistinguishable from zero at all voltages.

which displays traces at various negative potentials. Transitions between the three substates are observed at all of these voltages, but the probability of observing the individual states varies with voltage. At low potentials, the U state is observed almost all the time, and the M and D states rarely. As voltage is made increasingly negative, the probabilities of observing the M and D levels increase progressively, at the expense of the U level probability. At moderately positive voltage (e.g. +40 mV), transitions from the U state into the M state are almost never seen (data not shown).

TABLE 1. SUBSTATE CONDUCTANCES UNDER VARIOUS CONDITIONS

(Single-channel fluctuation records were collected under the indicated conditions of ionic medium and applied voltage. Conductances of the U and M states were measured with respect to the closed state of the channel. Each datum represents the mean ± s.e.m. of 5–20 measurements. In all cases, the conductance of the D state was indistinguishable from zero, within 5 %.)

condition	γ_U/pS	γ_M/pS	γ_U/γ_M
150 mM Cl^- (−50 mV to +50 mV)	18.5 ± 0.5	9.4 ± 0.1	1.97 ± 0.08
150 mM Cl^- (−135 mV)	15.9 ± 0.3	8.1 ± 0.2	1.97 ± 0.09
500 mM Cl^- (−90 mV)	21.9 ± 0.2	10.9 ± 0.2	2.01 ± 0.06
150 mM Cl^- + 1 mM SCN^- (−75 mV)	9.4 ± 0.2	4.9 ± 0.1	1.92 ± 0.08
150 mM Cl^- + 1 mM SCN^- (−150 mV)	7.7 ± 0.1	3.7 ± 0.1	2.08 ± 0.09
150 mM Br^- (−140 mV)	6.1 ± 0.1	2.9 ± 0.1	2.07 ± 0.14

It is possible to measure the current–voltage relation (*I–V* curve) of the three substates over the entire voltage range, as shown in figure 4. Between −80 and +50 mV, the *I–V* curves for both the U and M states are linear, and the U state conductance (18.5 pS) is precisely twice that of the M state (9.4 pS). No discernible conductance can be detected in the D state. This equal spacing of the three conductance levels might be a fortuitous result of the particular conditions used in these traces. To test this possibility, the three conductance levels were measured under a variety of ionic conditions leading to differing absolute conductances of the substates. The results are shown in table 1. The substate conductances are raised at higher Cl^- concentration, and are lowered by several manoeuvres: by application of very high voltage (more than 100 mV), at which the *I–V* curves become sublinear, by adding 1 mM SCN^-, a reversible voltage-dependent channel blocker, and by substituting Br^- for Cl^-. Under all such conditions, in which the absolute conductances vary over a 3.8-fold range, the U state always displays twice the conductance of the M state, within about a 5% error, and the D state and closed-state conductances agree within 5%.

By analysing long-term records of the transitions in the channel, it is possible to measure quantitatively the time-averaged probabilities of the appearance of the three substates. A representative result is shown in figure 5. The frequencies f_i of appearance of the substates are seen to vary with voltage, as indicated qualitatively in figure 3. More importantly, at all voltages tested, these substate frequencies follow closely a binomial distribution:

$$f_D = (1-p)^2, \tag{1a}$$

$$f_M = 2p(1-p), \tag{1b}$$

$$f_U = p^2, \tag{1c}$$

where p is a single voltage-dependent parameter. In other words, if one of the substate frequencies is measured, the other two are accurately predicted by a binomial distribution.

This result, taken together with the equal conductance spacing of the three substates, immediately intimates that the substructure of the Cl^- channel is an expression of the independent opening and closing of *two* identical Cl^- diffusion pathways. In such a scheme, the parameter p would represent the probability of a single one of these pathways being in its conducting state; likewise, the U state of the channel would represent the simultaneous opening of *both* of these individual pathways, while the M and D levels would represent the mixed 'open–closed' and the 'both closed' states, respectively.

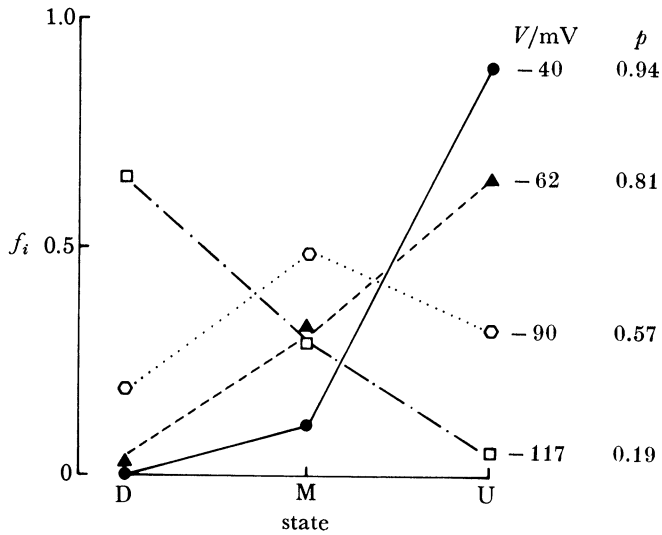


FIGURE 5. Substate frequencies are binomially distributed. Substate fluctuation records, collected as in figure 3, were used to calculate the long-term frequency, f_i, of the channel's appearance in state i (i = U, M, D). The closed-state intervals were not included in the analysis. Total time in each level was measured by hand, and frequencies were calculated by normalization to the total time of the record. For each voltage, 200–300 substate transitions were used. Points represent the measured frequencies, and lines connect theoretically expected values for a binomial distribution (equations (1)). These were calculated by taking the square root of the measured value of f_U as the binomial parameter p, and then calculating the expected values of f_D and f_M from (1 *a*) and (1 *b*). The good agreement between theory and experiment is representative of a larger body of single-channel data on this system; in the worst case encountered, there was a 15 % disagreement between measured frequency and the binomial distribution.

Two additional observations support such a picture. The first of these is that the binomial parameter p, extracted from the measured substate frequencies via (1), varies with voltage as expected for a simple two-state mechanism (Ehrenstein *et al.* 1970; Labarca *et al.* 1980), as shown in figure 6:

$$p(V) = [1+\exp(-zF(V-V_0)/RT)]^{-1}. \quad (2)$$

For the conditions of figure 6, the half-saturation voltage, V_0, is −95 mV, and the effective gating charge, z, is 1.1. Preliminary results show that V_0 depends on the pH of the *cis* side of the bilayer, such that the p–V curve shifts to the right under more basic conditions and to the left under more acid conditions. Also shown in figure 6 is the probability for channel opening via the *slow* gating process (broken curve), as determined previously. As voltage is made more negative, the probability of forming an open channel increases, but the channel, once open, dwells increasingly in the lower conductance substates.

The second supporting result concerns the stochastic laws obeyed by the substate fluctuations, i.e. the time distributions of the three levels. All the states are exponentially distributed in time (data not shown), as expected for *any* model in which a single level represents a single state of the channel. It is the particular behaviour of the mean dwell times in the three states that supports the specific 'binomial' model under consideration. Such a model must obey the rate laws embodied in the following scheme:

$$D \underset{\mu}{\overset{2\lambda}{\rightleftharpoons}} M \underset{2\mu}{\overset{\lambda}{\rightleftharpoons}} U, \tag{3}$$

where λ and μ are the 'fundamental rate constants' for opening and closing of the individual Cl^- diffusion pathways (Labarca *et al.* 1980). By measuring the mean dwell times $\bar{\tau}_i$ in the different levels, we can estimate λ and μ.

$$1/\bar{\tau}_D = 2\lambda, \tag{4a}$$

$$1/\bar{\tau}_M = \lambda + \mu, \tag{4b}$$

$$1/\bar{\tau}_U = 2\mu. \tag{4c}$$

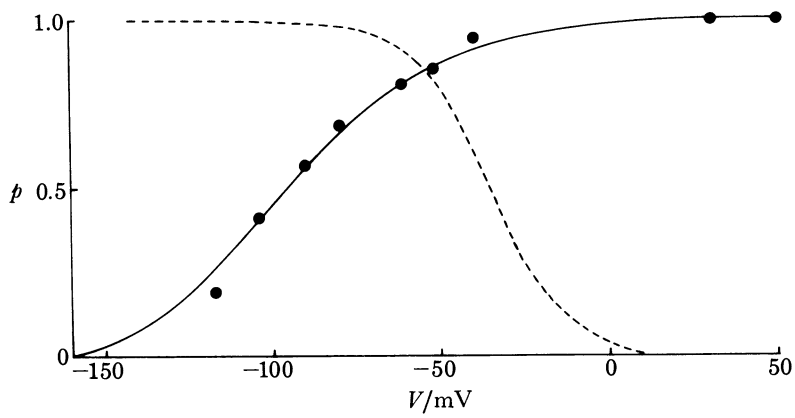


FIGURE 6. Probability–voltage curve. The binomial parameter p was measured from substate frequencies as in figure 5, and is plotted against applied voltage. The solid curve is drawn according to (2), with parameters given in the text. The broken curve shows the voltage dependence of the *slow* opening process, determined in previous work (White & Miller 1979; Miller & White 1980).

Also, we note that the binomial parameter p may be expressed in terms of the rate constants:

$$p/(1-p) = \lambda/\mu. \tag{5}$$

Therefore, in terms of the binomial model, the rate constants are overdetermined; four measurable quantities, $\bar{\tau}_D$, $\bar{\tau}_M$, $\bar{\tau}_U$ and p yield calculations of only two rate constants, λ and μ. In other words, we may measure λ and μ in two different and independent ways:

$$\lambda = 1/2\bar{\tau}_D, \tag{6a}$$

$$\mu = 1/2\bar{\tau}_U, \tag{6b}$$

and

$$\lambda = p/\bar{\tau}_M, \tag{7a}$$

$$\mu = (1-p)/\bar{\tau}_M. \tag{7b}$$

Figure 7 shows that the rate constants for opening and closing, measured either by (6) or (7), agree reasonably well, given the limited statistics accessible to hand-analysis of the stochastic data. Furthermore, the rate constants vary exponentially with applied voltage, as expected by a two-state gating model for the individual pathways (Labarca *et al.* 1980).

Discussion

Mere inspection of the single-channel fluctuation records of the *Torpedo* Cl^- channel leads to a four-state model of the channel, containing one 'closed' and three 'open' states. Transitions between the closed and open states are slow, in the range of seconds, and have been studied

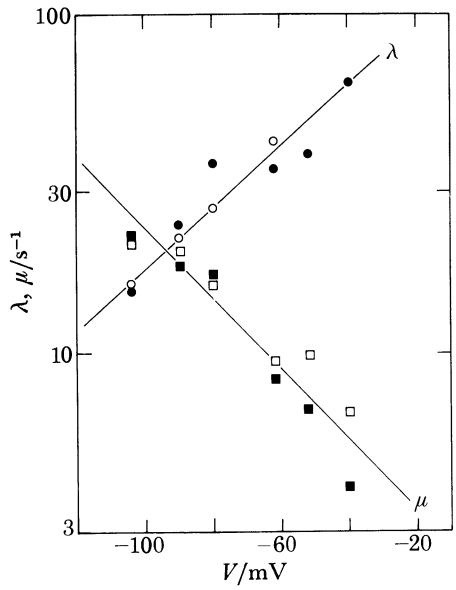
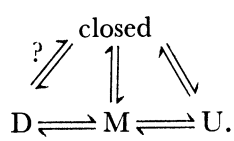


FIGURE 7. Voltage dependence of fundamental rate constants of fast gating. Substate fluctuation records were collected as in figure 5, and the mean dwell times in the three substates were measured by hand, using 50–300 dwell times for each calculation. Fundamental rate constants of opening, λ (circles), and closing, μ (squares), were calculated in two ways. Filled points: calculation via (6); open points: calculation via (7).

previously (White & Miller 1979, 1981*b*; Miller & White 1980). Transitions between the three 'open' states, U, M and D, are much faster, in the 10 ms timescale (at pH 7.4). Furthermore, preliminary results (not shown) demonstrate that the channel can enter the closed state from either the U or the M state. (A transition from the D to the closed state would be electrically invisible.) Therefore, a minimal scheme to describe the Cl^- channel is as shown here:



(8)

Integral membrane channels with multiple open states have been observed previously. The acetylcholine receptor channel (Hamill & Sakmann 1981), Ca^{2+}-activated K^+ channels (Marty 1981; Pallota *et al.* 1981; Latorre *et al.* 1982), the K^+ channel of amphibian sarcoplasmic

reticulum (Labarca & Miller 1981), the mitochondrial porin channel (Colombini 1979), even gramicidin A (Busath & Szabo 1981), all display more than a single conductance level when 'open'. The substructure of the *Torpedo* Cl^- channel is unique, however, in that it can be understood quantitatively in terms of a model involving two simple 'two-state' channels in parallel as the conducting unit (figure 8).

The evidence for such a model is strong. The substates are always equally spaced in conductance; the substate probabilities are binomially distributed; the transition probabilities

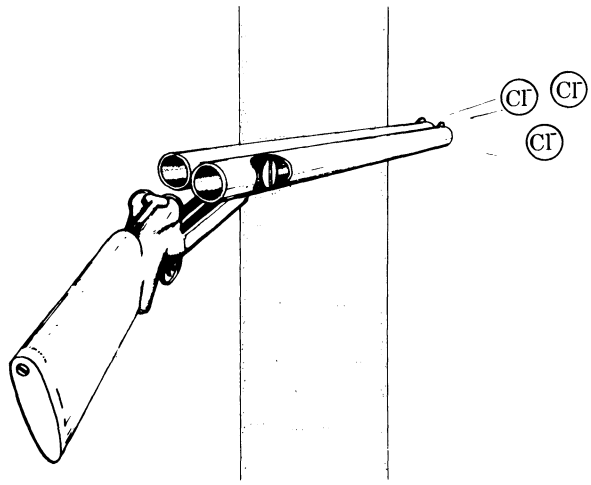


FIGURE 8. Cartoon of the 'double-barrelled Cl^- channel'. Each protomeric channel, or 'barrel', is assumed to contain a voltage-dependent gate fluctuating on a millisecond timescale. The channel's slow opening and closing process is represented by the cocking mechanism of the shotgun.

between substates follow kinetic laws demanded by such a model; both the long-term probabilities and the fundamental rate constants vary with voltage as expected for a two-state scheme.

We might ask why we do not consider that the rapid transitions between the three substates simply represent the simultaneous presence in the bilayer of two identical and distinct Cl^- channels. Indeed, there would be no way to reject this hypothesis if we observed only the rapid fluctuations between substates. It is the *slow* transitions into and out of the 'closed' state of the channel that allow us to eliminate such a picture. We *never* observe an open state of only the 'M level' conductance; the three substates always appear and disappear together. Thus, although the individual 'protomeric' channels undergo fast gating independently of each other, they are obligatorily coupled in a 'dimeric' structure by the slow gating process.

Why do we never see the three substates at highly positive voltages (figure 1)? The apparent 'single open state' at positive voltage is a consequence of the voltage-dependence of the fundamental rate constants of opening and closing, λ and μ (figure 7). If the variation seen in figure 7 continues out to high positive voltages, we would expect a U state mean dwell time at +90 mV of about 2 s, and mean dwell times in the M and D states of 0.6 and 0.3 ms, respectively, too short for detection here. Thus no special reasons need to be invoked to explain the persistence of the U state at high positive voltages.

The physiological significance of this substructure is unknown. It is reasonable to assume that the channel resides in the non-innervated-face membrane of the electroplax cell and is

responsible for the very high conductance required in this membrane to allow the high-current, high-voltage pulses generated by the fish in stunning its prey. But it is known that during such a pulse, the voltage across the non-innervated-face membrane does not change significantly (Bennett *et al.* 1961). The suggestion has therefore been made (White 1981) that because the electroplax organ evolved from skeletal muscle, a tissue containing a voltage-dependent Cl^- channel (Warner 1972), the voltage-dependent phenomena seen in the electroplax channel may simply be evolutionary debris without specific functions for the *Torpedo* itself. It is interesting to note that the reverse voltage-dependences of the fast and slow gating processes would lead to macroscopic Cl^- currents which upon prolonged depolarization from a highly negative potential would appear to be a fast activation followed by a slower 'inactivation'.

In working with planar bilayers, a constant worry of artefactual conductances plagues the experimenter, especially in a channel unstudied by electrophysiological approaches in the cell. The reproducible and specific behaviour extensively documented for this channel lay to rest worries about non-specific 'leak' artefacts, but the question remains: To what extent has the insertion of these channels into planar bilayers altered their characteristics? Two facts bear upon this question. First, a Cl^- conductance inhibitable by stilbene disulphonates has been shown to be present in the native *Torpedo* membrane vesicles (White & Miller 1981*b*), but the detailed characteristics of this conductance could not be studied by using the vesicle-flux techniques employed in that study. Second, it has recently been found that detergent extracts of these *Torpedo* membranes may be reconstituted by conventional methods into liposomes; these liposomes may be studied by the excised patch-clamp method (Horn & Patlak 1980), and we find that a Cl^- channel is reproducibly observed showing characteristics identical to those documented here (Tank *et al.* 1982). The fact that the Cl^- channels show the same behaviour regardless of the type of bilayer into which they are incorporated or of the method of insertion argues that this behaviour is not an artefact due to a particular way of assaying the channels.

Finally, it is worth noting that the 'dimeric' behaviour of the channel documented here is entirely novel; this type of substructure has never been seen in the many cation-specific channels that have been studied on the single-channel level. Since this Cl^- channel is the only anion-specific channel to be studied in detail, the possibility arises that certain fundamental structural differences may exist between anion and cation channels from higher organisms.

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